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SHORT COMMUNICATION

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Abstract Chemical signaling is a vital mode of communication for most organisms, including larval amphibians. However, few studies have determined the identity or source of chemical compounds signaling amphibian defensive behaviors, in particular, whether alarm pheromones can be actively secreted from tadpoles signaling danger to conspecifics. Here we exposed tadpoles of the common toad *Bufo bufo* and common frog *Rana temporaria* to known cues signaling predation risk and to potential alarm pheromones. In both species, an immediate reduction in swimming activity extending over an hour was caused by chemical cues from the predator *Aeshna cyanea* (dragonfly larvae) that had been feeding on conspecific tadpoles. However, *B. bufo* tadpoles did not detectably alter their behavior upon exposure to potential alarm pheromones, neither to their own skin secretions, nor to the abundant predator-defense peptide bradykinin. Thus, chemicals signaling active predation had a stronger effect than general alarm secretions of other common toad tadpoles. This species may invest in a defensive strategy alternative to communication by alarm pheromones, given that Bufonidae are toxic to some predators and not known to produce defensive skin peptides. Comparative behavioral physiology of amphibian alarm responses may elucidate functional trade-offs in pheromone production and the evolution of chemical communication.

Keywords Amphibian · Antimicrobial peptide · Bradykinin · *Bufo bufo* · Pheromone

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Introduction

Chemical signaling is very well known in aquatic predator–prey systems (Chivers et al. 1996; Kiesecker et al. 1999; Wisenden 2000; Pollard 2011). In aquatic amphibians, detection of alarm signals is a widespread strategy cueing defensive behavior (Hews and Blaustein 1985; Hews 1988; Lutterschmidt et al. 1994) but little is known about whether tadpoles actively secrete alarm pheromones signaling danger to conspecifics. Behavioral responses have been observed due to "disturbance signals", ammonium or ammonia from tadpole excretions (Kiesecker et al. 1999; Manteifel and Kiseleva 2011). Other studies have identified ions from dragonflies fed with tadpoles (Ferland-Raymond et al. 2010). Characterized amphibian pheromones are predominately peptides or proteins that can be detected by the vomeronasal or olfactory receptors (Apponyi et al. 2004; Woodley 2010).

Chemical alarm signals are typically categorized as kairomones if they originate from a predator or as pheromones if they originate from conspecifics (Schoeppner and Relyea 2005). Tadpoles typically respond to chemical alarm signals by trading off normal activities such as foraging for adoption of behaviors to reduce predation risk (Petranka 1989; Lima and Dill 1990; Werner and Anholt 1993). Tadpole prey can often identify alarm signals in

specific environments and avoid predators or locations labeled by a predator or locations labeled by other prey that have been alarmed or injured. The predator itself may be "labeled" while consuming or digesting the prey. In other words, unique chemical cues may be produced by the predator–prey interaction including digestion (Chivers and Smith 1998; Van Buskirk and Arioli 2002; Schoeppner and Relyea 2005, 2009; Hettyey et al. 2010). Predators that have been chemically labeled while eating tadpoles may present a predation risk detectable by conspecifics. This labeling may enable tadpoles to distinguish among low risk and dangerous predators irrespective of the novelty or historical occurrence of the predator. Thus, there are several advantages to detecting chemical cues originating from conspecifics rather than from predators (Chivers and Smith 1998).

The specific compounds that amphibians are able to secrete and detect are not well characterized. Several bioactive compounds in skin secretions are known to function in immune and in predator defense (Erspamer 1994; Daly 1998; Zasloff 2002). Chemicals stored in the dermal granular glands include peptides and proteins, lysozymes, aromatic amines, steroids, toxins, and free fatty acids. Discharge of amphibian granular glands is caused by stress or injury (rev. in Rollins-Smith and Woodhams 2012), and low-level secretions may be continuously present on the skin of adults (Pask et al. 2012). One abundant peptide found in some amphibian secretions is bradykinin (Conlon and Aronsson 1997; Conlon 1999; Chen et al. 2010). The role of bradykinin, a non-antimicrobial neuropeptide, is not fully understood. Bradykinin can activate gastrointestinal smooth muscle (Conlon 1999), and thus may be an important anti-predator defense compound. Almost nothing is known about the ability of tadpoles to actively secrete or detect these compounds.

Here, we test for immediate behavioral responses to predator cues in larval common toads *Bufo bufo* and common frogs *Rana temporaria*. We then repeat these experiments with *B. bufo* tadpole skin secretions that may contain alarm pheromones. We test both enriched and concentrated skin peptides and potentially volatile compounds that are secreted from tadpoles under stress.

Materials and methods

Animal husbandry

Four amplecting pairs of common toads, *B. bufo*, were collected near Schaffhausen, Switzerland (N47°42.065′/E8°35.633′) in March 2010, and kept in captivity overnight for egg collection. Egg clutches from common frogs, *R. temporaria*, were collected directly at the same pond. Tadpoles were reared for two weeks in outdoor artificial ponds (70 L plastic cattle tank) at the University of Zurich until they reached approximately 0.02 g (*B. bufo*) or 0.03 g (*R. temporaria*) at Gosner (1960) stage 26. The rearing tanks were provided with leaves and zooplankton to establish semi-natural conditions. Natural food in the tanks was irregularly supplemented with fish feed (Sera Spirulina Tabs; sera GmbH, Heinsberg, Germany).

Dragonfly larvae, *Aeshna cyanea* (hereafter "predators"), were caught at a pond near Zürich, Switzerland (N47°23.355′/E8°33.699′) and 20 individuals were kept separately in plastic cups containing 200 ml of water and small pieces of plastic mesh on which the predators could climb.

Predator cue experiments

Tadpoles were exposed to three different predator cue treatments: (1) water that formerly contained predators fed with conspecific tadpoles, (2) water that formerly contained unfed predators, or (3) control treatment with aged tap water. Sample sizes for each experiment are described in Table 1. Stage 26 *B. bufo* and *R. temporaria* tadpoles were distributed into small plastic tubs (31 × 21 × 11 cm) each containing 3 L dechlorinated tap water and placed on indoor shelving in a temperature uncontrolled room that approximated ambient late-spring conditions. Predators feeding on tadpoles were moved into approximately 150 ml fresh water the evening before the experiment was conducted, just after feeding. Each of the fed predators was provided with approximately 0.03 g of living *R. temporaria* or 0.02 g *B. bufo* tadpole three days before the experiment and the evening before the experiment. On the day of investigation, predators were removed from their cages and the water from 10 unfed predators was combined and diluted to 2.1 L. Similarly, water from 10 tadpole-fed predators was combined and diluted to 2.1 L. A third 2.1 L bottle contained aged tap water for the control. Bottles were color coded and experimenters were blind to the contents of the three bottles. From one of the three bottles, 100 ml treatment-water was added to experimental tubs containing tadpoles. Previous studies indicated that reduced activity was a typical response in *R. temporaria* and *B. bufo* to different predators (Van Buskirk 2001; Marquis et al. 2004). Thus, we calculated the proportion of individuals per tub that were actively swimming (moving the tail), inactive in water column (without moving the tail), or still (motionless on the bottom of the enclosure). A "snapshot" of tadpole behavior in each tub was observed immediately and at two later time points (1–5, 35–40, and 65–80 min), and tubs were observed sequentially to examine potential temporal dynamics of tadpole responses. Immediate behavioral responses are a logical gauge for

Table 1 Behavior of *Bufo bufo* or *Rana temporaria* tadpoles in four experiments tested by repeated-measures ANOVA

Effect	*N* (tadpoles per replicate)	*N* (replicates per treatment)	Wilks' Lambda		
			df	*F*	*P*
Bufo bufo and predator cues	8	20			
Behavior × Treatment			4, 112	2.664	**0.036**
Behavior × Time			4, 54	9.576	**0.000**
Behavior × Treatment × Time			8, 108	1.542	0.151
Rana temporaria and predator cues	5	10			
Behavior × Treatment			4, 52	2.585	0.063
Behavior × Time			4, 24	1.956	0.134
Behavior × Treatment × Time			8, 48	3.527	**0.003**
Alarm pheromone experiment 1. Treatments: enriched skin extracts, frozen skin extracts, bradykinin (0.25 μM), norepinephrine control (100 μM), water control					
	8	20			
Behavior × Treatment			8, 188	0.669	0.719
Behavior × Time			4, 92	2.536	**0.045**
Behavior × Treatment × Time			16, 281.7	0.765	0.725
Alarm pheromone experiment 2. Treatments: volatile skin extracts, norepinephrine control (100 μM), water control					
	8	20			
Behavior × Treatment			6, 150	0.643	0.695
Behavior × Time			18, 59	0.587	0.895
Behavior × Treatment × Time			54, 176.6	0.464	0.969

Predator cue experiments included treatment with water, or water containing cues from predatory *A. cyanea* (dragonfly larvae) that previously starved or fed on tadpoles. Alarm pheromone experiments examined responses of *B. bufo* tadpoles over time to enriched or frozen skin extracts from conspecifics, volatile skin extracts, or to synthetic bradykinin, norepinephrine, or water only controls (see "Methods" for treatment details). Significant effects are in bold

detection of chemical alarm cues indicating immediate predation risk, although long-term responses such as morphological or developmental shifts are also possible and have been previously reported (Van Buskirk 2001).

Responses to potential alarm cues

Tadpoles were exposed to one of five potential alarm cue treatments, each replicated 20 times. As above, groups of eight *B. bufo* tadpoles were distributed into plastic tubs, and researchers were blind to the treatments which included the following components diluted to 2.1 L in aged tap water, and distributed 100 ml per tub: (1) induced tadpole extract thawed just before the experiment, (2) enriched extract, (3) bradykinin (0.25 μM final concentration in tubs) of the synthetic peptide (purity 95 %; GenScript USA Inc., New Jersey), (4) norepinephrine (100 μM final concentration) control, (5) water control. The concentration of norepinephrine was known to be effective at inducing tadpole peptides in previous experiments. Bradykinin is thought to be a predator-defense compound capable of initiating a vomit reflex in some predators (Conlon 1999). The peptide is abundant in the skin at quantities up to 20–500 mg/g tissue in some species (Nakajima et al. 1979; Conlon and Aronsson 1997). Here we used 16.8 mg dissolved in 2.1 L as a "low dose" of bradykinin. Tadpoles in each tank were observed three times (as above) after adding the stimulus.

To collect potential alarm cues from stressed *B. bufo* tadpoles, five tadpoles were placed into each of ten 50 ml vials containing 40 ml of water with 100 μM norepinephrine (NE). After 15 min, tadpoles were removed and the water was pooled. Half of the water was frozen over night (= induced tadpole extract, see below). The other half was enriched by passing over C-18 Sep-Pak cartridges and concentrating to dryness by centrifugation under vacuum (= enriched extract, see below).

Responses to potentially volatile alarm cues

Tadpoles were exposed to potentially volatile alarm cues with a similar experimental design as described above except that the stimuli were prepared immediately before addition to the tadpole tubs. Treatments included: (1) induced tadpole extract, (2) norepinephrine control, (3) tadpole-water control, and (4) water control. For the first treatment, induced tadpole extract, three *B. bufo* tadpoles were added to 40 ml water in a 50 ml centrifuge tube with

100 μM norepinephrine. After 15 min, the induced secretions were immediately added to the experimental tanks. This minimized loss of volatile substances. For the norepinephrine control, water with norepinephrine was used as the stimulus. In preparing treatment three, three tadpoles were added to 40 ml pure water without norepinephrine for 15 min. This treatment may have caused stress and secretion of alarm cues. For the final treatment, water was used as a control. Tadpole behavior was observed in each tub every minute for 10 min in order to detect an immediate effect of potentially short-lasting volatile compounds that may be cue anti-predator responses.

Statistical analysis

Behavioral differences among treatments and over time were analyzed by repeated-measures ANOVA. Due to lack of sphericity (Mauchly's Test of Sphericity, $p < 0.05$), the Wilks' Lambda multivariate test of the within subjects effect is reported. Observations at the final time point were compared by standard ANOVA with Tukey pairwise post hoc comparisons. All statistical tests were performed in IBM SPSS Statistics 19.0 (SPSS Inc., Chicago, IL).

Results

Predator cue experiments

Exposure to cues from dragonfly predators previously fed on tadpoles cued behavioral responses in *R. temporaria* and *B. bufo* tadpoles (Fig. 1; Table 1). The reduced activity response was detectable for over an hour after exposure to the stimulus. There was a greater reduction in *R. temporaria* than in *B. bufo* activity, and common toad tadpoles were not particularly active even in control treatments (approximately 10 % actively swimming, Fig. 1). Although *B. bufo* did not show a significant response to cues from starved predators, *R. temporaria* did (Fig. 1).

Responses to potentially volatile and non-volatile alarm cues

We detected no significant behavioral response of *B. bufo* tadpoles to potential alarm cues (Table 1). Similarly, no immediate behavioral responses were detected upon exposure to potentially volatile compounds within skin secretions (Table 1). In all *B. bufo* experiments except the time-limited volatile cue experiment, there was a significant interaction between behavior and time (Table 1) such that tadpoles showed a tendency to sink and remain still at the initiation of cues (regardless of treatment), and became more active through time.

Discussion

In aquatic predator–prey systems, kairomones, or chemical cues released by the predator are often detected by prey (Kats and Dill 1998). We confirm previous findings that tadpoles of *B. bufo* and *R. temporaria* can react to kairomones (Marquis et al. 2004). Responses to kairomones

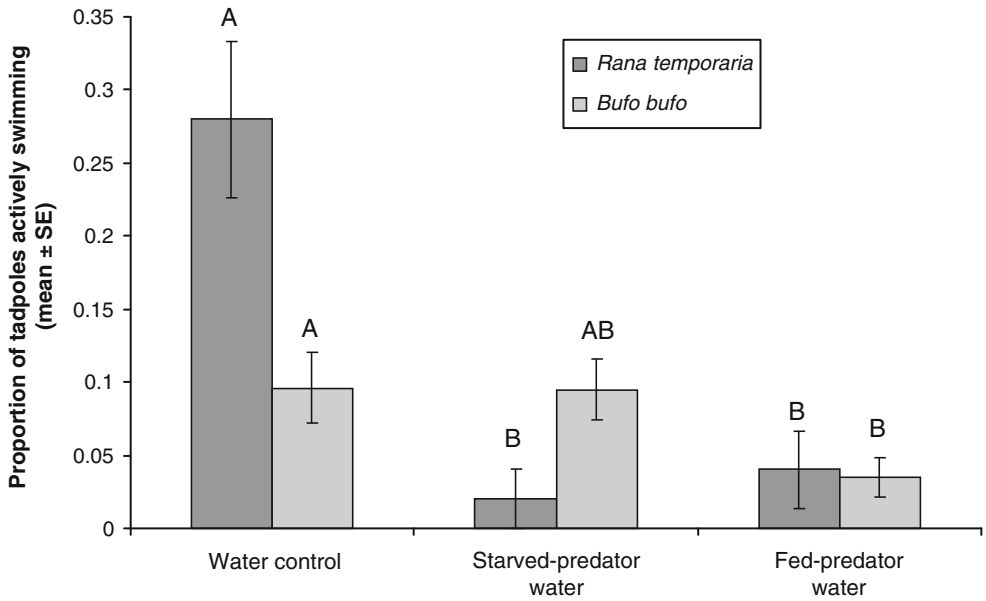

Fig. 1 Proportion of actively swimming tadpoles upon exposure to water from different alarm cue treatments. Letters above bars indicate homogenous subsets based on ANOVA and Tukey post hoc tests; *R. temporaria* and *B. bufo* tested separately. Behavior from the third observation period is displayed, beginning just over an hour post-exposure to potential alarm cues

were greater than responses to actively secreted alarm pheromones, whether enriched or volatile, produced by *B. bufo* tadpoles. Tadpoles of both species significantly reduced their activity when exposed to cues of fed predators (*A. cyanea*), and *R. temporaria* tadpoles exposed to cues of starved predators reduced activity an intermediate amount (Fig. 1). This reduction in swimming activity was observed immediately and over an hour after cue exposure. Thus, it is not surprising that long-term exposure of tadpoles to predator cues can affect feeding efficiency, growth rates, and time to metamorphosis among other characteristics (Petranka 1989; Lima and Dill 1990; Werner and Anholt 1993; Van Buskirk 2001; Van Buskirk and Arioli 2002). Because predators fed on conspecifics often elicit greater behavioral responses compared to starved predators, a secondary role of compounds released from prey during capture, or during digestion has been suggested (Hews and Blaustein 1985; Hews 1988; Lutterschmidt et al. 1994; Fraker et al. 2009).

The active secretion of alarm pheromones upon detection or attack by a predator is not as clearly indicated in tadpoles as in some other systems. Chemical alarm systems occur in a large variety of taxa, including fishes (Smith 1992), gastropods (Stenzler and Atema 1977), and echinoderms (Snyder and Snyder 1970). In Ostariophysan fishes, specialized epidermal cells contain alarm substances (Schreckstoff) that can only be released upon rupture of the cells (Chivers et al. 2007). Attraction of additional predators upon attack may provide greater opportunities for prey to escape (Chivers et al. 1996; Wisenden 2000). These alarm cues are not actively secreted upon stress or danger. Alarm substances from injured toad tadpoles are avoided by conspecifics (Hagman and Shine 2009). We tested whether uninjured tadpoles of *B. bufo* could actively secrete alarm pheromones that trigger immediate changes in swimming behavior in conspecifics, similar to the changes we observed upon exposure to predator cues. However, *B. bufo* tadpoles did not detectably alter their behavior upon exposure to any of the potential alarm cues tested. These included conspecific skin secretions, skin secretions concentrated and enriched for the peptide component, the abundant predator-defense peptide bradykinin, and potentially volatile cues collected from stressed conspecifics. While negative data can be difficult to interpret, we demonstrate that chemicals signaling a previous predation event induced a stronger effect than potentially available actively secreted alarm pheromones on the defensive behavior of *B. bufo* tadpoles. This does not preclude the possibility of low level responses to secreted alarm signals or behavioral responses different than those examined here. However, similar behaviors may be expected because of the adaptive value of responding to predation or alarm signaling predation risk. Thus, responses to predation risk in *B. bufo* are physiologically underpinned by detection of kairomones and injured conspecifics, ammonia alarm excretions (Manteifel and Kiseleva 2011), and to a much lesser extent, secreted alarm pheromones. Behaviors specific to each type of cue deserve further study.

Common toads are known to produce toxins such as bufadienolide (Flier et al. 1980) that make eggs, larvae, and adults unpalatable to some predators (Griffiths and Denton 1992; Semlitsch and Gavasso 1992). Bufonidae are also not known to produce antimicrobial peptides, even in adult stages (Conlon et al. 2009). Thus, production and reception of secreted alarm pheromones is more likely to occur in species without these alternative anti-predator defenses, or in species that are capable of secreting skin peptides in the tadpole stage. Comparative studies are needed to understand the role of chemical communication among tadpoles.

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